Spring 5-10-2014

Role Model Fathers Or Deadbeat Dads? A Study of Peromyscus Paternal Behavior

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ROLE MODEL FATHERS OR DEADBEAT DADS?
A STUDY OF PEROMYSCUS PATERNAL BEHAVIOR

By

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Submitted in Partial Fulfillment
of the Requirements for
Graduation with Honors from the
South Carolina Honors College

May 2014

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Thesis Summary

Paternal behavior is a complex and diverse phenomenon that is poorly understood when considered alongside maternal behavior and interactions. Paternal behavior is relatively common in species of birds and fish, but it is much more of a rarity when one considers mammalian species. It is estimated that only 3-5% of mammalian species are naturally paternal, and many of these are species of rodents and non-human primates. A common way to study paternal behavior is by using a comparative approach, which takes advantage of naturally occurring differences between closely-related species. Comparative models between paternal and non-paternal species of rodents are frequently used in research, such as those between voles and *Peromyscus* mice. In the *Peromyscus* model, *Peromyscus californicus*, a monogamous and paternal species, is compared to *Peromyscus maniculatus*, a promiscuous and non-paternal species. There are other species of *Peromyscus,* such as *Peromyscus polionotus* (PO) that have many benefits for use in research but have not had their paternal behavior characterized explicitly. This study makes use of a comparative approach between the BW and PO species in order to determine whether PO males exhibit paternal behavior. We hypothesize that BW males will show less paternal interaction with the pups and that PO males will exhibit distinct paternal behavior in comparison. The results from this study will be the first documentation of the nature of PO males' parental investment.

Mated pairs of 12 BWs and 10 POs were used for this study. The females were removed from the cage, the nest was disturbed, and the behavior of the male was filmed for 10 minutes following the disruption of the nest. An array of pup-directed and non-

pup-directed behaviors were scored, and the total durations of the behaviors were analyzed for species differences. The analysis revealed that PO males spent significantly more time grooming the pups than the BW males. PO males also spent significantly less time jumping and burrowing during the testing period than the BW males. When the durations of all of the close proximity and pup contact behaviors were considered together, the average amount of time spent exhibiting pup-directed behavior was significantly higher in PO males. Conversely, BW males spent significantly more time exhibiting behaviors with no pup contact and non-approach behaviors (non-pup-directed behavior). The average duration of time spent huddling with the pups was not significantly different across species, but this behavior did show significant differences in duration as a function of pup age. As the pups increased in age, both PO and BW males spent more time huddling with them.

The findings from this study indicate that PO males spend more time than BW males on specific paternal behaviors, such as grooming, and also spend more time exhibiting pup-directed behaviors as a whole. This supports the hypothesis that PO males would exhibit more paternal behavior in comparison to BW males. However, it is not clear how PO males compare to other males that are accepted as the "paternal standard" for current animal models. The question of whether or not POs can be considered to be a paternal species cannot be definitively answered without more comparative studies that also take known paternal species, such as *Peromyscus californicus*, into consideration. It is reasonable to conclude that POs do exhibit some forms of paternal interaction and they are therefore more paternal than BWs, the accepted "non-paternal standard". The exact

place of the PO species on the spectrum of *Peromyscus* paternal behavior, however, remains to be seen.

Abstract

Paternal behavior is a largely understudied and poorly understood topic, especially in mammalian species. Many current mammalian models for paternal behavior use a comparative approach, taking advantage of natural differences in behavior between closely related species. This study compared paternal behavior in two rodent species, namely *Peromyscus maniculatus* (BW) and *Peromyscus polionotus* (PO). PO rodents have been shown to be monogamous, but there have been no studies of their paternal behavior at this time. 10 PO males and 12 BW males were filmed in their home cage for a 10 minute period following initial disturbance of their nest and removal of the female from the cage in order to compare their paternal interactions and care of the pups following the disturbance. PO males spent significantly more time grooming the pups than the BW males. PO males also spent significantly less time jumping and burrowing during the testing period than the BW males. Overall, time spent exhibiting pup-directed behavior was significantly higher in PO males. Conversely, BW males spent significantly more time exhibiting non-pup-directed behavior. These results support the hypothesis that PO males exhibit more paternal behavior compared to the BW males. However, further study would be required in order to determine if PO males meet the standards of currently accepted paternal models, like *Peromyscus californicus* or *Microtus ochrogaster*, and if they can therefore be considered to be a "truly paternal" species.

Introduction

Paternal investment is a phenomenon that is highly diverse across species. These differences in paternal behavior arise due to differences in the ecological requirements for survival of the species as well as differences in mating styles (promiscuous, monogamous, polygamous, etc.) **[20].** Social monogamy is defined by characteristics such as the exhibition of male parental behaviors and the presence of alloparenting behaviors in sexually naïve animals of the species **[4]**. Paternal investment in offspring care is observed in many species of fish and birds, as well as some species of rodents and non-human primates. In fact, 90% of bird species exhibit some form of paternal investment, and one hypothesis is that in ancestral avian species, offspring care was performed exclusively by males and has since evolved in a unidirectional fashion to the currently observed forms of parental care **[33]**. However, paternal investment is significantly less common in mammalian species, with only 3-5% of all mammalian species exhibiting naturally paternal behavior **[7]**.

Regardless of species, paternal behaviors and paternal investment is an understudied topic. Most research about parental behaviors focuses on the mechanisms and behaviors involved in maternal care, partly because the majority of this research is on mammalian species where paternal care is notably rare. In some typically non-paternal rodent species, males can exhibit facultative care in order to ensure that their offspring survive **[25]**. Paternal care refers to those behaviors that a male exhibits naturally and under normal conditions in the protection and care of his offspring. Facultative care is different from these paternal behaviors, however, because their presence is contingent on

the state of the environment or the offspring at the time. For example, males that only show paternal behaviors when the female is absent or the offspring are in danger or dying could be characterized as exhibiting facultative paternal care because they only show these behaviors when absolutely necessary. However, especially in biparental species, both the maternal and paternal aspects of care are needed to facilitate the growth, development, and viability of offspring, working together in a complementary relationship **[20]**.

It is possible that varying levels and types of paternal involvement could lead to variable impacts in the cognitive and emotional development of the offspring, which would be significantly important for human applications and considerations. One of the reasons for developing animal models for parental and paternal involvement is to better understand the mechanisms and behaviors involved with these types of care and to pinpoint the effects that different types of care have on the offspring. Some research regarding human paternal involvement and its impact on child development has been conducted. For example, human studies have revealed that children that are raised in an unstable caretaking environment (separated from their biological father or raised with a step-father) are more susceptible to unusual levels of stress hormones and a higher frequency of illness **[8]**. Negative interactions, especially the implementation of harsh disciplinary methods, with a father that is in close proximity to the child throughout early development is a strong predictor of the development of aggressive tendencies in the child later in life **[35]**. Paternal influence on child development is not restricted to negative impacts, however, and several studies have indicated that paternal involvement can contribute greatly to successful, favorable development as well **[20]**.

When studying paternal interaction and influence in humans, a confounding factor that limits the generalizability of the results is the spectrum of categorization of what constitutes "paternal behaviors". Studies have used different measures of physical availability and proximity, paternal warmth toward offspring, and other measures that can be perceived as methods of "paternal investment" in the offspring's survival (planning, provisioning, financial support, etc.) in order to characterize levels of paternal interaction **[20]**. Can measures of human paternal behavior be directly derived from current maternal models, or should gender roles and modern cultural expectations be taken into account when characterizing the parental investment that can be contributed by human males? Environmental factors and cultural expectations clearly influence paternal behavior in humans. Examining paternal behavior in non-human animals allows the investigation of the neurobiology of paternal behavior to be separated from the cultural factors and expectations of gender roles that can complicate similar studies in humans. Animal investigations also allow a means of standardizing different types of paternal interactions and interpreting the impact of paternal care on offspring development within a simpler social context. Rodents are commonly used for developing animal models of parental behaviors due to the fact that many different rodent species have known mating styles (monogamous vs. promiscuous), making it easier to predict their parental tendencies and whether males of the species will participate in the care of the offspring. The animal models allow for a detailed examination of the neural, neurochemical, and genetic basis of paternal behavior without the complicating factors of culture and gender role expectations.

When mammals undergo the transition of becoming a parent, their "naïve brain" undergoes changes that cause a shift of focus from self-preservation and increased likelihood of future reproduction to a focus on social attentiveness and care of their offspring **[23]**. When this transition occurs in female rodents, "on-the-nest" behaviors such as retrieval of offspring, nursing, and grooming as well as other "off-the-nest" behaviors like foraging are acquired by the parent in order to better equip them to care for their offspring **[23]**. As discussed earlier, male rodents may or may not exhibit paternal behaviors or may only exhibit paternal behavior under certain circumstances. Male rodents may develop different on/off-the-nest behaviors than females and may not fit the commonly referenced maternal template. For example, research done in dwarf hamsters suggests that male rodents' primary role is aiding with the thermoregulation of newborn pups and that absence of the male adversely impacts offspring survival **[36]**. This could mean that males primarily develop crouching and huddling behaviors over other forms of acquired parental behaviors.

Studies in rats have shown that the medial preoptic area (MPOA) of the hypothalamus plays an important role in the acquisition of maternal behaviors during the maternal transition because this area acts as an integration point, allowing associations to be made between the prefrontal cortex and areas of reward perception, such as the ventral tegmental area and the nucleus accumbens **[26]**. It makes sense that reward networks are involved in the maternal care pathway, as females would be more likely to continue to care for their young and expend the energy necessary to help them survive if they experience some type of reward-like motivation as a result of pup care. Studies in rats have documented that pup interaction increases dopamine release in the ventral striatum

[13] and that lesions to the ventral tegmental area (VTA) and dopamine antagonism in the nucleus accumbens and VTA both interfere with appropriate maternal care **[14, 18]**. While the role of these reward pathways in paternal behavior has not been explicitly studied, it is possible that these pathways may contribute a neural component to paternal care activation.

One of the most commonly studied areas of paternal behavior is the role and influence of hormones that may impact paternal behaviors in males. A wide array of studies of human and rodent models of virgin, "expecting", and parental males have shown that there are changes in the levels of many hormones, such as oxytocin, prolactin, vasopressin, and testosterone. In gonadectomized male rats, the implantation of estrogen into the medial preoptic area (MPOA) has been shown to induce maternal behavior **[31]**. This supports the idea that the MPOA is involved in the induction of parental behaviors and also the idea that estrogen, a hormone involved in pregnancy in females, is involved in maternal behavior. One finding that is applicable to the induction of paternal behaviors is from a study done in gerbils, which are known to be monogamous. Males that were pair-housed with their mates and pups for a long-term period had elevated serum levels of prolactin during and following pregnancy and showed rising levels of testosterone during pregnancy which sharply fell off following offspring birth **[3]**. Oxytocin, a hormone that is associated with birth and lactation in maternal animals and also with reducing responses to fear stimuli, has been shown to be elevated in monogamous *Peromyscus californicus* fathers when their mate is pregnant compared to both naïve males and parental males **[11, 19]**. Another hormone that may be involved in paternal responses is vasopressin, which is involved in social recognition and pair bonding and may play a role

in preventing mice fathers from committing infanticide once their offspring are born **[15]**. Many of the functions of the hormones that have been described could be linked to the parental changes or paternal behaviors that emerge when a male reaches fatherhood. As stated here, there are several different rodent models that point to a major contribution of the endocrine system in the induction of paternal behaviors. However, it is important to remember that hormone measures may be subject to time dependencies, brain region differences, and species differences as well.

Comparative vole studies have supported the idea that there is a strong endocrine component to the acquisition of parental behaviors. In one study which compared paternal prairie voles (*Microtus ochrogaster*) to non-paternal montane voles (*M. montanus*), oxytocin gene expression was elevated in females (but not males) of both species but vasopressin gene expression was increased only in male and female prairie voles (compared to sexually naïve controls) **[34]**. These findings suggest that oxytocin may be a major hormone that acts in the induction of maternal behavior and that paternal behavior may rely on a separate hormone, vasopressin. Interestingly, there are other effects that have been observed in sexually naïve prairie voles that have been exposed to a pup compared to naïve prairie voles with a control stimulus. These voles, following the pup stimulus, showed transient rapid increases in plasma concentrations of oxytocin, no changes in serum vasopressin levels, and attenuation of corticosterone release following handling **[19]**. Corticosterone is typically associated with stress following handling, so this attenuation may mean that the pup exposure, even to a naïve male, suppresses the fear response. Studies done in *Peromyscus* (which will be discussed later in this introduction) suggest that paternal animals have an enhanced capacity to deal with stress

and anxiety **[2]**, so this may be due to the hormonal effect that pup exposure has on parental males. These findings also suggest that the vasopressin increase may be a change that is unique to truly parental males and not a change that occurs due to pup exposure alone.

A comparative approach is often used in order to take advantage of naturally occurring differences between species and to analyze differences in their behavior. In analyzing paternal differences between species, the behaviors of interest can be grooming, crouching, and retrieval of offspring as well as passive association behaviors, which measure time spent on, around, and away from the nest **[20]**. The current study will analyze both of these types of behaviors. Comparative paternal behavior studies typically compare a known paternal species to a closely related non-paternal species, and much of the current comparative literature on paternal behavior involves Peromyscus and vole models.

Comparative behavioral studies conducted in voles have yielded very interesting observations about the parental behavior of these animals. Measures of paternal behavior in all prairie voles (*M. ochrogaster*) are high and do not show group differences between males that are parental, males that have had social exposure, and males that have had no social contact **[17]**. In addition, findings from other vole studies have shown that sexually naïve prairie voles engage in spontaneous offspring care and exhibit alloparenting behaviors such as grooming, retrieval of offspring, and assuming an "arched back" nursing posture over the nest **[19, 28]**. These findings suggest that male prairie voles may be "constitutionally adapted for fatherhood" due to their high rates of paternal behavior, regardless of parental experience **[20]**.

Peromyscus are also used in comparative approaches to study and understand parental behaviors. The two most commonly compared species are *Peromyscus californicus* (California mouse), which is a monogamous species with paternal behavior, and *Peromyscus maniculatus* (BW), which is a promiscuous, non-paternal species. The paternal behaviors of the California mouse are very similar to the maternal behaviors observed in this species and include behaviors such as grooming and crouching over the nest, while the BW males exhibit almost no parental responses in the presence of pups **[10]**. In a comparative behavioral study, BW males engaged in avoiding a distressed pup stimulus, while California males with parenting experience showed pup-directed behaviors in response to the same stimulus **[22]**. Studies in California mice have shown that both estradiol and testosterone promote paternal behavior in male mice **[32]**. Estradiol is a steroid that has been linked to the induction of maternal behavior, and testosterone is known to aromatize into estradiol via aromatase enzymes. The fact that Trainor et al. (2002) found that both estradiol and testosterone induce paternal behavior in male California mice is exciting because it suggests a potential endocrine and physiological mechanism by which the paternal behavior is induced. Based on studies done in rats, rhesus monkeys, and Syrian hamsters, male mammals have increased aromatase activity in the medial preoptic area (MPOA), the medial amygdala, and the bed nucleus of the stria terminalis, indicating that these brain regions could be where testosterone is aromatized to estradiol to induce paternal changes **[16, 29, 30]**. Interestingly, this correlates with the previously discussed rat study in which injections of estrogen into the MPOA of gonadectomized males induced maternal behavior **[31]**. This suggests that these brain regions may be important in aromatizing testosterone, which has

been shown to be elevated throughout pregnancy in gerbils **[3]**, to estradiol, thereby inducing the paternal response.

Peromyscus studies have also shown that parental males have increased plasma concentrations of prolactin and oxytocin when compared to expecting fathers and virgin males, although testosterone levels across these three groups are not significantly different **[11, 12]**. This is an instance in which a comparative study to a non-parental species, like the BW mice, would be very useful in order to determine what hormone changes are contributing to the paternal behavior seen in the California mice. Behaviorally, parental male California mice exhibit enhanced spatial memory and suppressed anxiety responses when exposed to a stress stimulus, resulting in fewer breaks in their behavioral chains, when compared to pup-exposed virgins and naïve virgin males **[2, 9]**. These adaptations would have clear advantages for parental males, allowing them to perform their offspring care duties more efficiently and giving them enhanced emotional resilience.

When considering the rat, vole, Peromyscus, and other rodent and mammalian models that have been presented, it is evident that there are equivocal findings regarding the changes observed in and influences of various hormones and neurotransmitters in the induction of parental, and specifically paternal, behaviors. Some species indicate that the role of oxytocin or vasopressin are important in the induction of paternal behavior **[25, 34]**, while others show no changes in these hormones in relation to the induction of paternal care **[11]**. Studies that compare paternal species' hormone levels to non-paternal species' hormone levels are needed in order to illuminate exactly what chemical changes are present that distinguish a paternal from a non-paternal animal. This would have been

helpful, for example, in the *Peromyscus californicus* study in which levels between virgin, expecting, and parental males were compared **[11, 12]**, because it would have given a context for the changes seen between different parental states. Combining findings from studies that use different models and species can make it difficult to make generalizations about the true underlying mechanisms and behaviors that are being observed. For example, the discussion about the potential paternal involvement mechanism for estradiol and its aromatization to testosterone drew information from studies done in many different animal models, including California mice, rats, rhesus macaques, gerbils, and Syrian hamsters.

It is possible that the induction of paternal behavior may be very species-specific, with only a few common areas that overlap across mammalian species. However, it is important to know which mechanisms are common and which differ across species in order to fully understand paternal behavior. For this reason, it is important to develop comparative studies that answer these questions. Much of the paternal behavior literature that exists for Peromyscus is done comparatively between *Peromyscus californicus* and *Peromyscus maniculatus*. However, there are other species of Peromyscus that are understudied that have particular advantages for research. *Peromyscus polionotus* (PO) is one of these understudied species, but one that would be very advantageous to study due to the fact that a full genome analysis exists for this species. Another Peromyscus species that could be advantageous to study is the F1 hybrid species, which result from a genetic cross between a BW male and a PO female. These Peromyscus could be used to study any sex-linked traits that may be correlated with paternal behavior. Species like the POs and California mouse are frequently used to investigate hypotheses concerning stress

response and social behavior **[6]**, therefore understanding the behavior and mechanisms involved with their paternal behavior could help illuminate the mechanisms involved in other social interactions. At this time, no behavioral studies exist that examine the paternal behavior of *Peromyscus polionotus* (PO) or the F1 hybrids. The current study will examine and compare the paternal behaviors of BW and PO *Peromyscus* and establish the paternal behavior across these species.

We hypothesize that the *Peromyscus maniculatus* males will show less paternal interaction with their offspring since this has been observed in previous research **[10, 22]**. *Peromyscus polionotus* is known to be monogamous, but no studies to date have been conducted to determine if this species is paternal. However, paternal behavior is typically seen with monogamy **[20]**. We hypothesize that the *Peromyscus polionotus* males will exhibit distinct paternal behavior, and the confirmation of this hypothesis will be the first concrete evidence to support the idea that this species is paternal, confirming long-held assumptions to this effect.

The current study will be the first study to document the paternal behaviors of the BW and PO *Peromyscus* in a comparative approach. This study will also lay the behavioral groundwork to enable further investigations to be conducted on the mechanisms of paternal behavior induction and how these mechanisms may differ across these species.

Methods

Subjects

Animals that were used in this study were housed at the *Peromyscus* Genetic Stock Center at the University of South Carolina at Columbia, South Carolina. The animals were housed under a 16-8 hour light-dark cycle. In this study, twelve mated pairs of Peromyscus maniculatus (BW) and ten mated pairs of Peromyscus polionotus (PO) were used. All animals had free access to food and water and were housed in mated pairs with pups. Pups that were used in this study ranged from 5-30 days of age. Age of male and female and number of pups and age of pups was noted.

Behavioral Testing

Behavioral testing for this study took place within the animals' home cage. During behavioral testing, the adult female was removed from the home cage and placed into a clean cage, so that interaction with the pups would be limited to the male subject. Excess shavings were removed from the cage to facilitate observation of the male at all times during testing. These shavings were returned to the home cage after filming.

The nest was disturbed such that the pups were removed from the established nesting area. The male's response was filmed for 10 minutes, after which the female and excess shavings were returned to the home cage.

Behavioral Scoring

The recordings of males' behavior were scored by multiple blind observers and inter-rater

reliability was 0.90. The Observer Program (Noldus) was used for scoring. Scoring of behaviors was conducted using continuous sampling in order to record the precise moment that each behavior occurred. Behaviors were grouped into three over-arching groups, and the behaviors within each group were considered to be mutually exclusive events. The behavior groups were Distance, Contact, and Non-Contact. The behaviors within each group, along with their operational definitions, are given in Table 1 (See Tables and Graphs, page 34).

Results

The total durations of the following scored behaviors were averaged for each subject and analyzed for species differences: retrieval, grooming, sniffing, huddling, burying, approach, on nest, jumping, back-flipping, burrowing, and non-approach. In addition to these behaviors, two additional variables were defined – pup-directed behavior and non-pup-directed behavior. Pup-directed behavior is an additive variable that encompasses all of the behaviors that are considered to be directed by the male toward the offspring and includes the total durations of retrieval, grooming, sniffing, huddling, burying, approach, and on nest. Non-pup-directed behavior, on the other hand, includes the sum of the durations of jumping, back-flipping, burrowing, and non-approach. These behaviors were the ones that were considered to be actions that were not directed toward offspring care and attentiveness. Pup-directed behavior and non-pup-directed behavior were also analyzed for species differences. Tables 2 and 3 give the proportion of males demonstrating a particular behavior and the duration and standard errors of the mean of each behavior across species respectively.

Levene's Test for Equality of Variances was used to analyze the variance of all of the variables. The test indicated that the following variables did not have equal variances across species: grooming duration, sniffing duration, burying duration, jumping duration, back-flipping duration, and burrowing duration. For these variables, t-tests not assuming equal variances were used. For the rest of the variables, equality of variance was assumed in the t-tests.

PO males groomed the pups more than BW males ($t(20) = -2.678$, $p = 0.025$). PO males spent less time jumping during the testing period than BW males ($t(20) = 2.228$, $p = 0.045$). PO males also spent less time than the BW males burrowing under the shavings in the home cage ($t(20) = 4.009$, $p = 0.002$). Overall, there was significantly more pup-directed behavior observed for the PO males than there was for the BW males ($t(20) = -3.382$, $p = 0.003$). Conversely, significantly more non-pup-directed behavior was observed in the BW males compared to the PO males ($t(20) = 3.167$, $p = 0.005$). Figures 1, 2 and 3 show the relative amounts of behaviors in both pup-directed and non-pup-directed behaviors across species. Variables that were not significantly different between species were the following: retrieval duration, sniffing duration, huddling duration, burying duration, approach duration, on nest duration, back-flipping duration, and non-approach duration.

In addition to analyzing the species differences in average duration of time spent for each behavior, the impact of pup age on duration of behaviors was analyzed. For the pup age-dependent analysis, the tested pairs were separated into 2 age groups: males with pups less than or equal to 12 days of age and males with pups greater than 12 days of age. The number of males in each condition is shown in Table 4. Two-way Analyses of Variance (ANOVA) with species and age of pups as between factors were used to analyze total durations of the various dependent variables. The only behavior that showed significant differences in total duration as a function of pup age was huddling ($F(1,18) = 15.38$, $p = 0.001$). As the pups aged, both BW and PO males spent more time huddling with them. These data are shown in Table 5 and Figure 4. Finally, there were no effects of time of pairing of the male and female parents on any of the variables.

Discussion

One way to study paternal behavior is to conduct comparative behavioral studies, which take advantage of the natural differences between the paternal behaviors of different species. *Peromyscus maniculatus* and *Peromyscus californicus* have both been studied extensively and their species classification based on male involvement have been characterized as non-paternal and paternal, respectively. However, *Peromyscus polionotus* has not had the nature of its paternal behavior studied and documented. The results from this study shed some light on this subject. Because there has been so little research conducted on the nature of paternal behavior (as compared to maternal behavior), the maternal behavior template is one of the things to which the results of this study can be compared. Typical maternal behavior in rodents and mice usually involves behaviors such as nest building, nursing, crouching on or over the pups, retrieval of the pups, and licking or grooming the pups. It is important to remember that paternal behaviors may not fit the maternal template, and so these behaviors may not be the only ones that can be used to differentiate between a paternal and a non-paternal species.

In the current study, PO males spent more time grooming their pups than BW males. The fact that PO fathers spent so much more time on average grooming their pups than the BW fathers is an interesting and important finding because it is one of the behaviors that coincide with the maternal behavior template that is generally accepted for rodents and mice. The significant species difference in the duration of these behaviors is one indication that PO males are more paternal in nature than BW males.

Another behavior that showed significant species differences in average total duration were burrowing and jumping, with PO males spending less time doing these behaviors compared to BW males. In general, jumping in *Peromyscus* is associated with stress and is considered to be a stress response or avoidance behavior. The fact that PO males showed significantly less jumping than BW males during the testing period is interesting because this may indicate that PO males experience an attenuation of their stress response as a result of pup exposure, similar to the stress attenuation results that were observed by Bardi (2011) in *Peromyscus californicus*. Another possibility could be that PO males are less likely to experience fear or stress in general when they are in close proximity to pups. Studies by Lambert (2013) have already shown that non-paternal BW males engage in avoidance behaviors when placed in a context with a distressed pup. It may be that this avoidance behavior is a constitutive trait of non-paternal males, whether the pup stimulus is indicative of distress or not. This would explain why the BW males show higher average durations of jumping when compared to POs – they may be experiencing a fear response and be trying to get away.

The same type of discussion could be used to speculate about why PO males spent less time on average burrowing under the home cage shavings than BW males during the testing period. Burrowing could be associated with some types of nest building behavior or could be interpreted as an avoidance behavior. Additional studies that look more closely at nest building behavior and its correlates would be required in order to specifically disentangle the burrowing observed in this study from nest building, avoidance, and other exploratory behaviors. However, in the context of these observations it would seem that burrowing would be more likely to be associated with

avoidance of the pups after the nest had been disturbed. If this is the case, then the increased average duration of burrowing behavior exhibited by BW males compared to PO males could, like the jumping results, be indicative of increased stress and anxiety in the BW males in response to pup exposure. Ethical issues have been raised about whether pup exposure is actually a source of stress for BW males. While this has not been explicitly confirmed or rejected experimentally, some argue that if this is the case then BW males should not be housed with their pups in order to reduce additional stress factors from their environment.

It was surprising that there were no other scored behaviors that showed differences in total duration across species, especially the behaviors like On Nest, Sniffing, Retrieval, and Huddling. As discussed previously, On Nest and Retrieval are two behaviors that were scored that correlate to behaviors typically exhibited by maternal females. None of the PO males participated in retrieval of the pups, and only 2 out of 12 BW males briefly showed this behavior. These data suggest that this particular behavior is one that is rarely observed in male subjects, because it was observed so little in both species in this study. This could mean that paternal males do not participate in retrieval of the pups at all and that this is one point at which the two templates of expected parental behaviors diverge.

The average total durations of On Nest behavior between the two species approached significance, but was ultimately not a significant finding in this study. Intuitively it would seem that paternal and non-paternal animals would show differences in this behavior, and so the fact that this behavior was not significantly different across species could be an indication that PO males are not paternal, contrary to the original

hypothesis. It could also be that the sample size used in this study was too small to detect a significant difference for this behavior between the two species. Future studies could be conducted with larger sample sizes for each species in order to determine if On Nest behavior, another behavior which would correspond to the "maternal template", is in fact a behavior that differs across species. Sniffing and Huddling behaviors were also considered to be Contact behaviors, indicative of paternal attentiveness and care, and so it was surprising that these did not differ in their average total durations across species as well.

Even though Huddling did not show significant species differences, it was interesting to see that there were significant differences in Huddling duration as a function of pup age. As the pups increased in age, the average total duration of Huddling increased as well. There could be many reasons for this significant change in behavior. Postnatal day 12 is approximately the time that *Peromyscus* pups begin to have fur, and this could aid in social recognition of the pups by the father as another animal of his own species. If this is the case, then the father would no longer view the pups as a novel or foreign object in his environment, but rather as an equal member of his species. This would result in reduced fear and anxiety, especially in the BW males, and could manifest itself as increased total duration of huddling with the pups as a form of social interaction. Another option is that as the pups age and mature, the males huddle more with them in order to maintain heat and their own body temperature. This explanation would have fewer social implications.

Even though some of the behaviors did not show significant species differences as was expected, there were 2 more findings that have large implications for the conclusions

that we can draw about the paternal behavior of these two species. When all of the pup contact behaviors, Approach, and On Nest behaviors were grouped together and defined as Pup-Directed Behavior, it was found that PO males spent significantly more time than BW males exhibiting Pup-Directed Behavior, while conversely BW males spent significantly more time than PO males showing Non-Pup-Directed Behavior. While these variables of Pup-Directed and Non-Pup-Directed Behavior are not direct indicators of specific paternal behaviors (or lack thereof) for these species and may not be a direct or clear indication of whether or not a particular species is paternal versus non-paternal, they do show how the majority of the duration of the testing period was spent on average for each species. From these data, it can be concluded that PO males show increased paternal attentiveness and higher levels of paternal behavior relative to BW males. Based on models that are currently used in research, BWs are typically viewed as a non-paternal species. Therefore, it can be concluded that PO males show higher levels of paternal behavior and are at least moderately paternal, supporting the hypothesis that was presented for this study that PO males would show distinct paternal behavior compared to BW males, who would show non-paternal tendencies as observed in other studies.

There are still several questions that remain to be answered with regard to the question of the distribution of paternal tendencies across species of *Peromyscus*. Because the current study was comparative, the data obtained was able to indicate that PO males are relatively paternal with respect to BW males. However, this does not mean that this species completely meets the criteria to be termed a truly paternal species that is suitable for research on paternal mechanisms and social interactions. The next step for the investigation into this question would be to conduct another comparative study, this time

comparing the PO males to an established paternal species model, such as *Peromyscus californicus*. It could be that the PO males fall somewhere in between the two established extremes of the paternal behavior spectrum that is currently accepted for *Peromyscus* species, rather than being on the same level with the "truly paternal" extreme. This cannot be confirmed or rejected with certainty, however, without an additional comparative study to test this hypothesis.

Another future direction that can be pursued in this area of research is to investigate the paternal behavior of the F1 hybrids in a comparative approach with BW, PO and *Peromyscus californicus* males. As stated in the Introduction, studies involving the F1 hybrids could be used to illuminate any sex-linked factors that influence paternal behavior. Anecdotal evidence from the laboratory of Dr. Michael Felder suggests that the F1 males may show a distinct and discernable level of paternal behavior. One reason for this is the fact that even pair-housed males show extensive nest-building behavior. Another reason is that when the nest is disturbed, it is the males, not the females, which crouch over the nest and protect the pups, while the females have been observed to be the ones that run in circles in the cage without attending to the pups at all following cage disturbance. This suggests that there may be some change that occurs during the hybrid cross that imparts increased parental tendencies to the F1 males. However, these anecdotal observations would need to be studied formally in order to determine if and where F1 males fall within the spectrum of paternal behavior that is emerging for *Peromyscus*. In the original design of this study, F1 males were meant to be observed in addition to the BW and PO males. However, due to their lack of availability, these

observations and analysis would be suitable for inclusion in another comparative study in the future.

The results of this study support the hypothesis that PO males exhibit distinct paternal behavior compared to BW males, a closely related non-paternal species. The finding that on average more than half of the duration of the testing period for PO males was spent engaged in some type of Pup-Directed Behavior, whereas on average more than half of the testing period of BW males was spent engaged in Non-Pup-Directed Behavior, was the strongest result that led to this conclusion. Other results from this study, such as the significant species difference in average total Grooming duration, suggest that there are some behaviors exhibited by males that fall in line with correlated maternal behaviors. The absence of significant species differences in Retrieval duration and On Nest duration, however, also indicate that there are some ways in which the behaviors observed in paternal males may be very different from the common behaviors expected from maternal females. It seems that there are clear differences that separate maternal from paternal behavior in certain aspects. This means that there are differences in the underlying neurobiological pathways, not only between paternal and non-paternal species, but also between males and females within the same species, leading to these differences in parental behaviors. Once a clear, comparative spectrum of paternal behavior for *Peromyscus* can be established, studies that illuminate these neurobiological mechanisms can be used to better understand paternal behavior induction as a whole and also what types of neurobiological and neurochemical differences are inherent to differentiate paternal and non-paternal mammalian species.

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Tables and Figures

Table 1: Scored Behaviors and Operational Definitions

This table lists all of the behaviors that were scored for the films and the definitions of those behaviors

Behaviors and Operational Definitions	
Distance Group	
On Nest	Male is sitting on the pup or pups or is crouched over them in an arched back posture. Male is not jumping or back-flipping onto the nest.
Approach	Majority of male's trunk enters a 3 cm radius area of pup/pups.
Non-Approach	Majority of the male's trunk is located outside of the 3 cm radius area surrounding the pup/pups. Male jumping or back-flipping is always counted as non-approach regardless of distance.
Contact Group	
Huddling	Male joins the grouping of pups and huddles in a group along with them or under them. Male is not above the nest with an arched back posture.
Grooming	Male is licking and grooming the pups.
Retrieval	Male is moving the pups within the home cage. This behavior is characterized by grasping the pup by the skin of the neck or back and lifting it off of the ground.
Sniffing	Male participates in sniffing the pup(s), but does not initiate grooming behavior.
Burying	Male buries pups beneath shavings of the cage.
Non-Contact Group	
Jumping	Male exhibits jumping behavior at any point within the cage.
Back-flipping	Male exhibits back-flipping at any point within the cage.
Burrowing	Male is burrowing beneath the shavings of the cage. This is not considered to be a pup-directed behavior, regardless of his proximity to the pups during burrowing.

Table 2: Simple Proportions of Behavior Occurrence

This table lists all of the scored behaviors and gives a simple proportion of the number of males of each species that exhibited the behavior.

Behavior	BW Proportion	PO Proportion
Retrieval	2/12	0/10
Grooming	3/12	8/10
Sniffing	10/12	9/10
Huddling	7/12	7/10
Burying	2/12	3/10
Approach	12/12	10/10
On Nest	7/12	8/10
Jumping	7/12	3/10
Back-flipping	2/12	0/10
Burrowing	12/12	3/12
Non-Approach	12/12	12/12

Table 3: Average Total Durations and Standard Errors of Behavior Variables

This table lists the calculated average total durations and standard errors for all behavior variables for each species.

	BW		PO	
Behavior	Average Total Duration (seconds)	Standard Error (seconds)	Average Total Duration (seconds)	Standard Error (seconds)
Retrieval	2.2	1.9	0.0	0.0
Grooming	1.9	1.2	81.7	29.8
Sniffing	27.6	9.4	12.8	2.6
Huddling	40.5	17.6	104.8	36.4
Burying	0.6	0.5	6.3	4.9
Approach	157.6	15.1	154.3	30.5
On Nest	14.6	7.6	48.2	15.4
Pup-Directed Behavior	245.0	33.6	408.1	34.2
Jumping	32.4	12.7	3.4	3.0
Back-flipping	43.4	29.7	0.0	0.0
Burrowing	125.6	30.0	5.0	3.1
Non-Approach	176.8	22.8	210.6	32.5
Non-Pup-Directed Behavior	378.1	36.4	218.9	33.5

Table 4: Distribution of Pup Age Groups

This table shows the number of males of each species whose pups fell within the designated age groups for the pup age analysis.

Pup Age Group	BW	PO
Pups less than or equal to 12 days of age	5	4
Pups greater than 12 days of age	7	6

Table 5: Average Huddling Duration Differs by Pup Age

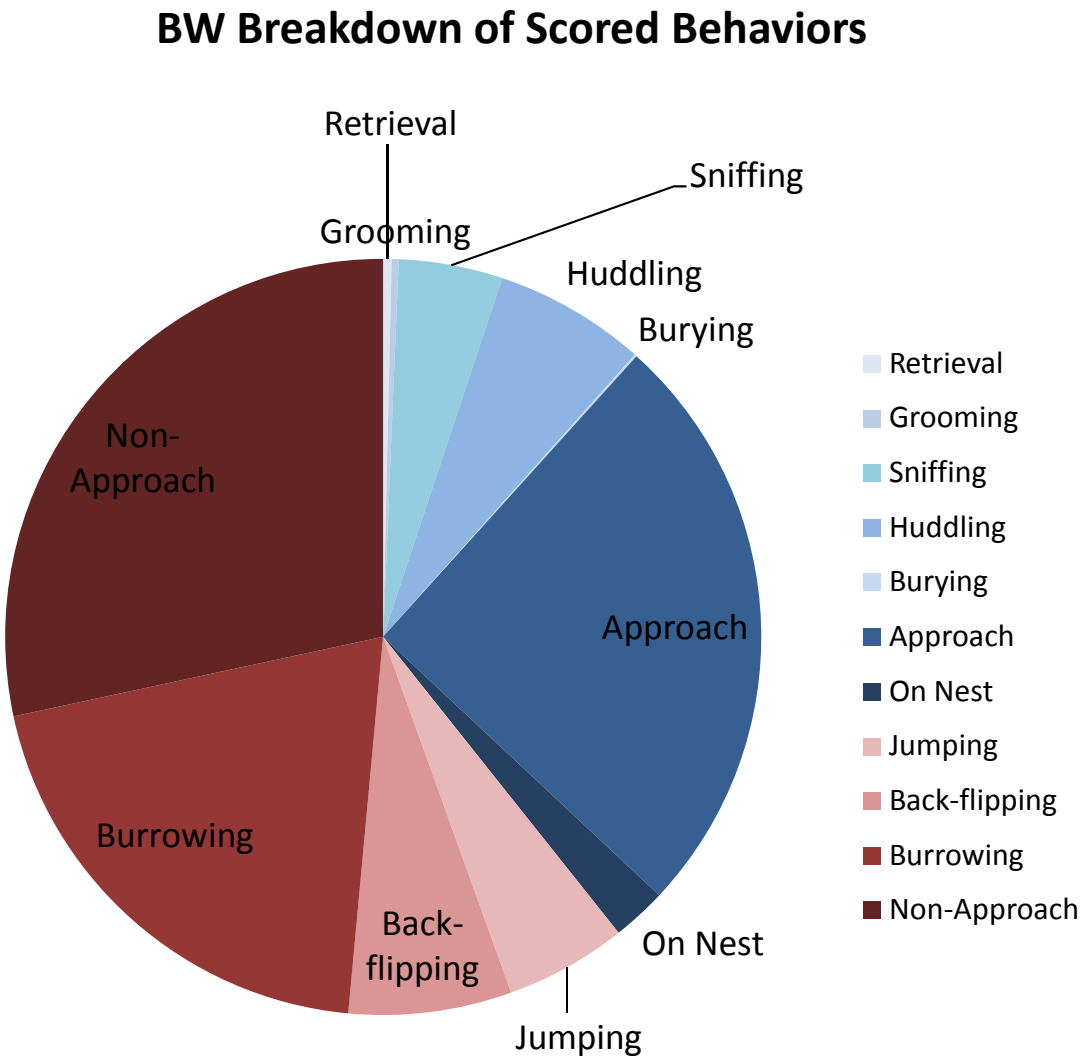
This table gives the mean and standard errors of the mean of the average huddling duration observed for each species as a function of pup age.

	Young Pup Average Total Duration (seconds)	Young Pup Standard Error (seconds)	Old Pups Average Total Duration (seconds)	Old Pups Standard Error (seconds)
BW	0	0	69.38	25.21
PO	7.47	7.47	169.62	42.90

Figure 1: Average Breakdown of Scored Behaviors

A. Graph of the average duration of scored behaviors relative to one another for BW males. B. Graph of the average duration of scored behaviors relative to one another for PO males. For both graphs, Pup-Directed Behaviors are shaded in blues, while Non-Pup-Directed Behaviors are shaded in reds.

A.



B.

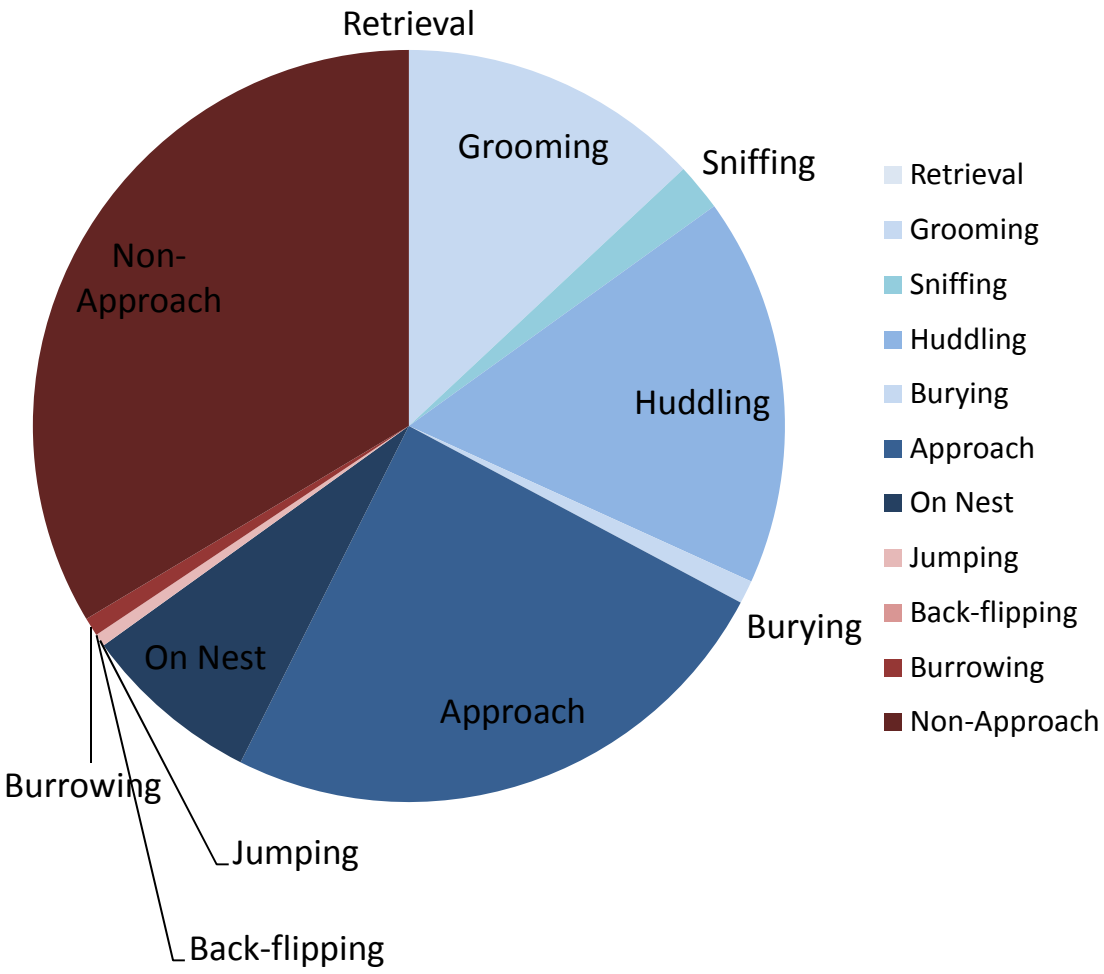
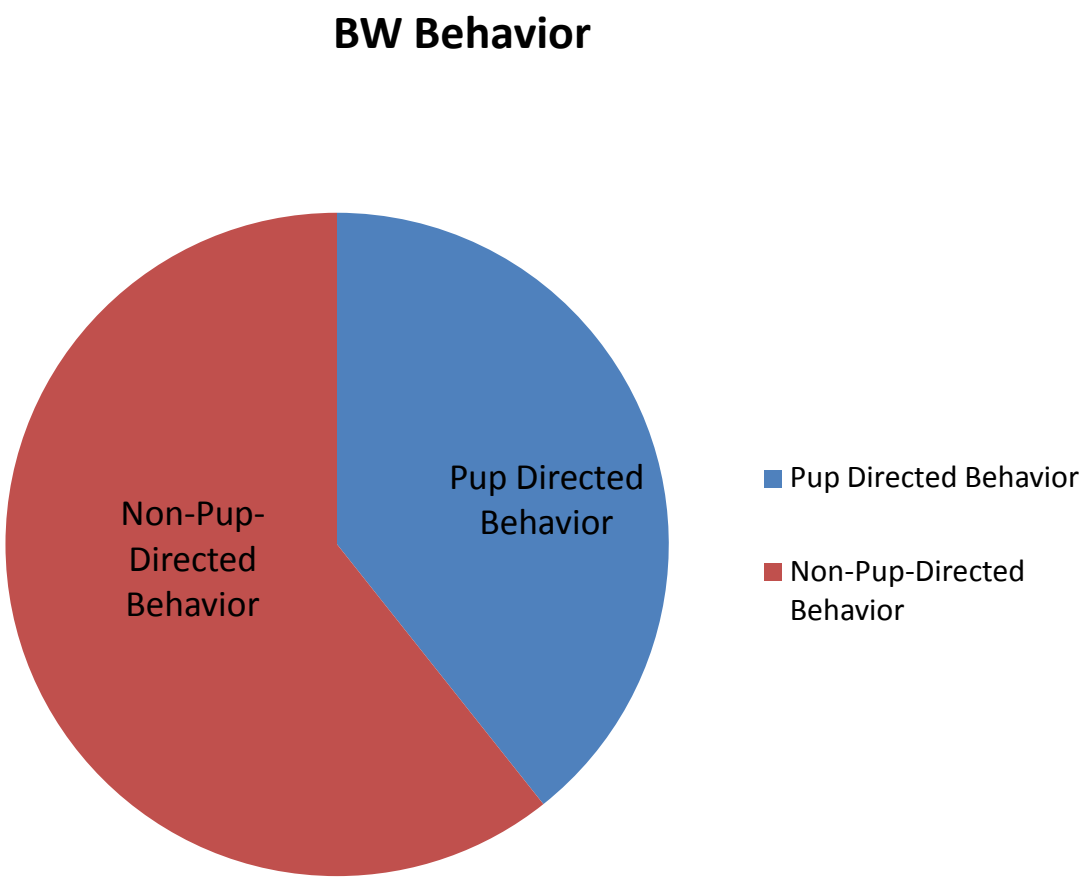

PO Breakdown of Scored Behaviors
Retrieval
Grooming
Sniffing
Huddling
Burying
Approach
On Nest
Jumping
Back-flipping
Burrowing
Non-Approach
Non-Approach
Burrowing
Back-flipping
Jumping
On Nest
Approach
Burying
Huddling

Figure 2: Relative Durations of Pup-Directed and Non-Pup-Directed Behaviors

A. Graph showing the relative durations of pup-directed and non-pup-directed behaviors for BW males. B. Graph showing the relative durations of pup-directed and non-pup-directed behaviors for PO males.

A.



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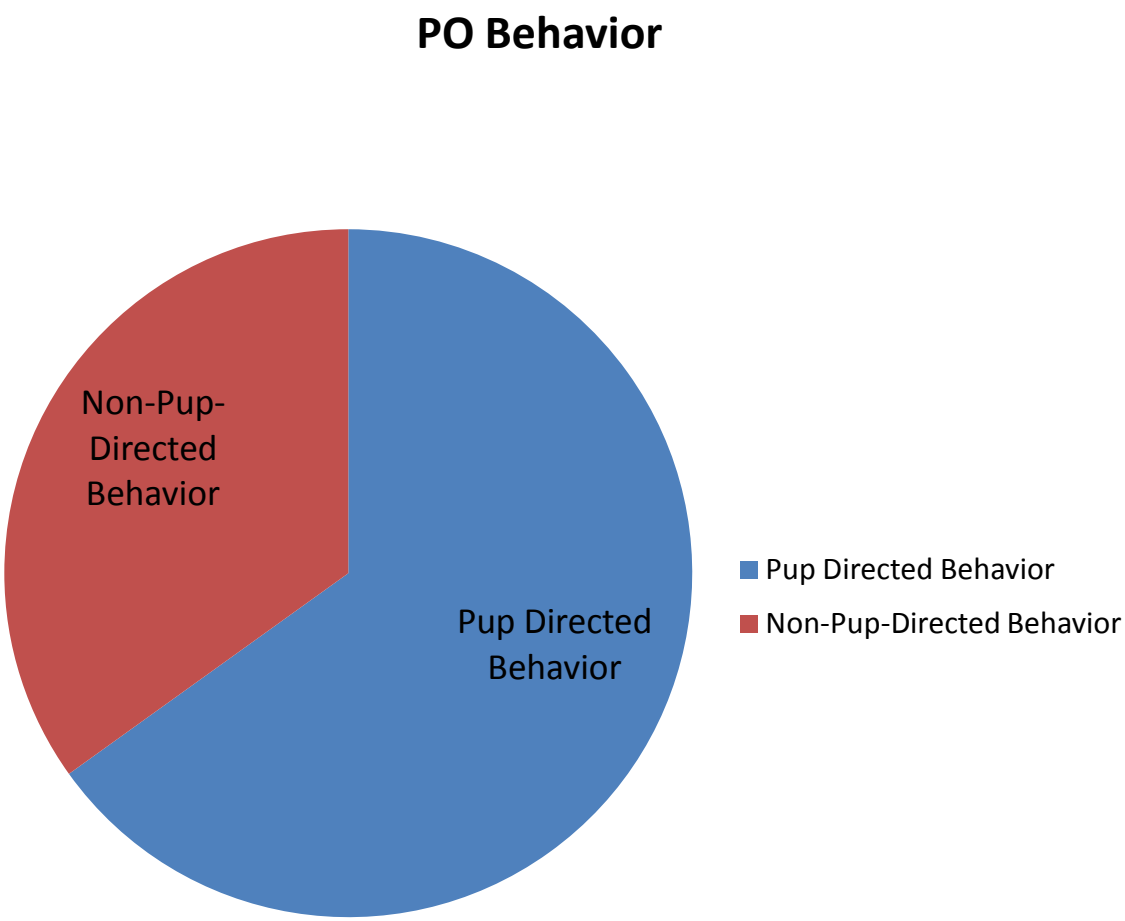

PO Behavior
Non-Pup-Directed Behavior
Pup Directed Behavior
Pup Directed Behavior
Non-Pup-Directed Behavior

Figure 3: Significant Species Differences in Total Duration of Paternal Behaviors

The behaviors in this graph are the ones that showed significant species differences in their total duration during the testing period. Behaviors that did not have significant species differences, namely On Nest and Huddling, are also shown in this graph. On Nest behavior approached significance ($t(20) = -2.058$, $p = 0.053$). Huddling did not show significant differences between species, but did show significant differences as a function of pup age (see Figure 4).

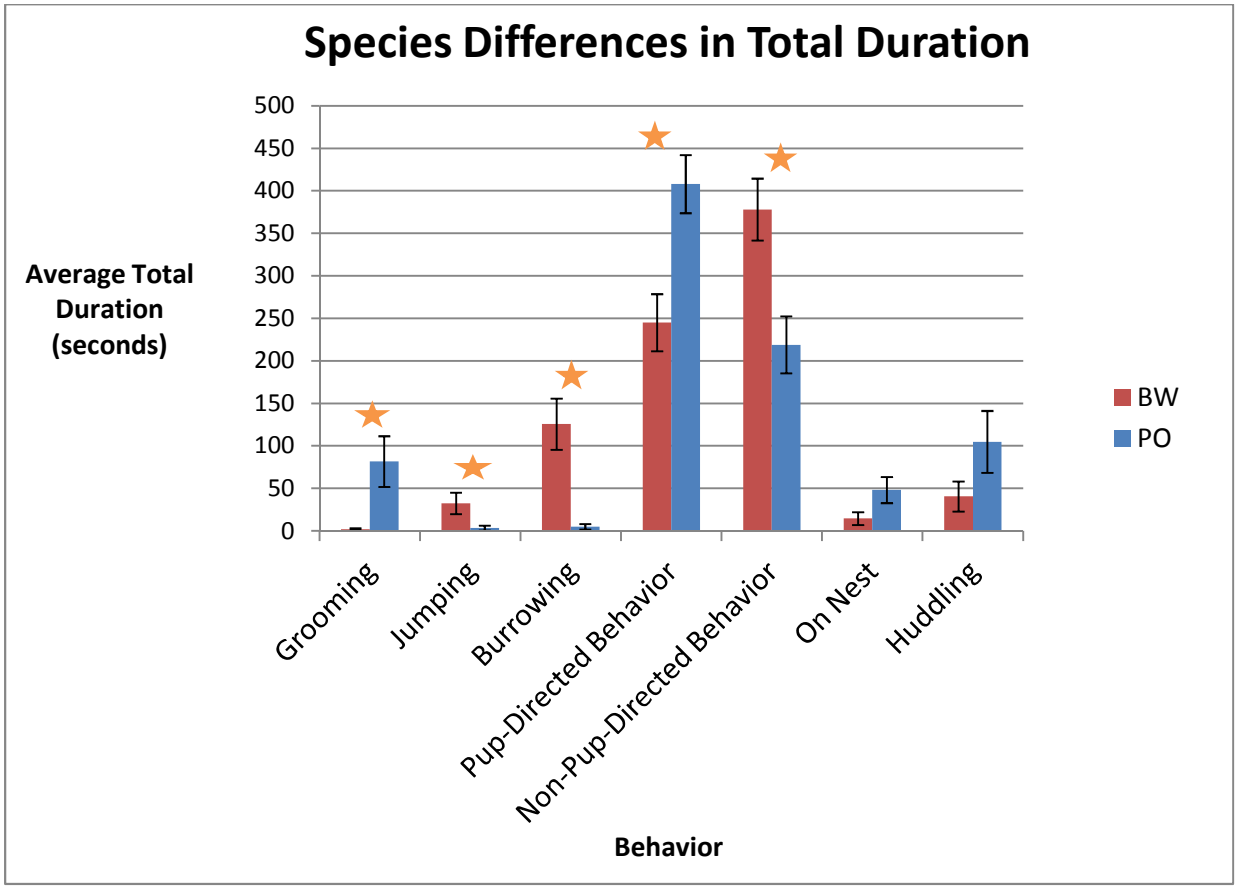
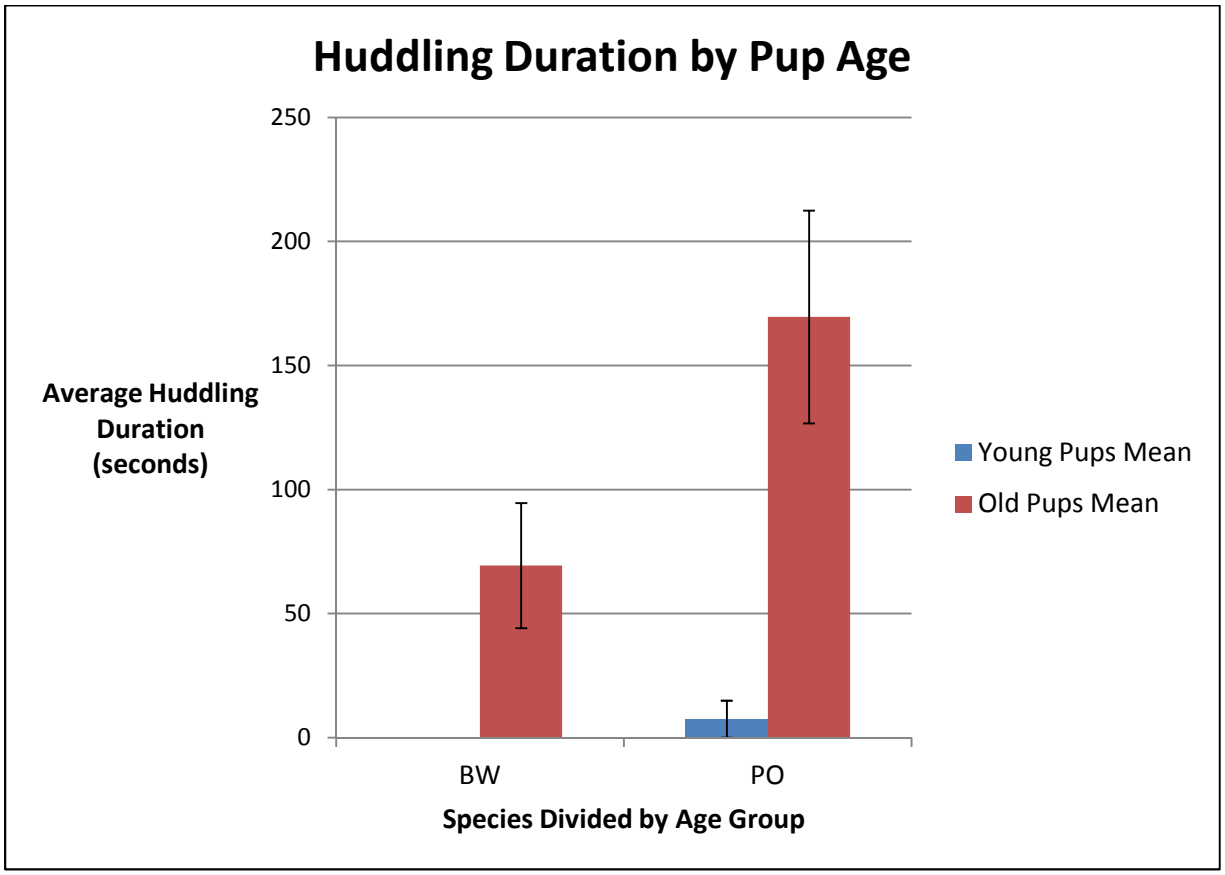


Figure 4: Average Huddling Duration As a Function of Pup Age

This graph shows the average huddling duration observed for each age group within each species. There is a clear increase in huddling duration for both species as the pups increase in age ($F(1,18) = 15.38$, $p = 0.001$).



Acknowledgements

Special thanks to the following people and institutions for all of their help and support. The successful completion of this research project and Honors Thesis paper could not have occurred without their aid.

Dr. Sandra Kelly, Thesis Director

Dr. Michael Felder, Second Reader

Peromyscus Genetic Stock Center at the University of South Carolina

Mrs. Janet Crossland

Members of the Kelly Lab: Amy Perkins, Victoria Macht, Daniel Wood, Anna Capps, Azissa Singh, and Tessa Crawford

Tyler Spires